

their localization in the gut or the body are not known.

Almost half of the residues in eggs are unmetabolized *trans*- and *cis*-permethrin in the yolk. The remainder is a great variety of metabolites in the yolk and white including most of those also detected in the excreta. Residues of permethrin-derived products in yolk reach peak levels 3 or 4 days after those in white in a similar manner to the metabolites of other xenobiotics (Andrawes et al., 1972; Davison, 1976; Paulson and Zehr, 1971). Radio-carbon from dermally applied ^{14}C -methylene-labeled (1*R*)-*cis,trans*-permethrin reaches maximum yolk levels at the fifth day after treatment (Hunt et al., 1978) in agreement with the present findings on oral administration of the individual *trans* and *cis* isomers.

The ease of permethrin hydrolysis and oxidation in hens suggests that rapid detoxification may contribute to the relative insensitivity of hens and other birds to pyrethroid poisoning (Casida, 1973; FMC Corporation, 1977).

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Supplementary Material Available: Complete tabulation of individual unidentified metabolites including amounts, TLC R_f values, and HCl cleavage products (2 pages). Table I of this paper utilizes this information in abbreviated form. Ordering information is given on any current masthead page.

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Toxicity of *Alternaria* Metabolites Found in Weathered Sorghum Grain at Harvest

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No evidence of toxicity was observed in rats or chicks when *Alternaria* metabolites alternariol monomethyl ether (AME), alternariol (AOH), and altenuene (ALT) were fed for 21 days at levels up to 24, 39, and 10 $\mu\text{g/g}$, respectively, which were about six times as great as those found in heavily weathered sorghum grain at harvest. Two of four isolates of *Alternaria alternata* were toxic when corn-rice-*Alternaria* culture constituted half the diet for chicks and rats. Although all the isolates produced AME, AOH, and ALT, the two that also produced tenuazonic acid and altertoxin I were lethal. Tenuazonic acid was not found in any of the 12 samples of weathered sorghum grain analyzed by gas chromatography.

When grain sorghum, *Sorghum bicolor* (L.) Moench, is exposed to wet weather before harvest, the seeds are often discolored by fungal growth. Two *Alternaria* metabolites, alternariol (AOH) and alternariol monomethyl ether

(AME), were found in Kansas sorghums in amounts that correlated with the degree of grain discoloration and with the number of rainy days during September and October (Seitz et al., 1975). The abundance of weathered, discolored sorghum in various areas in recent years has caused concern about possible toxicity to livestock.

Christensen et al. (1968) reported that 53 of 60 isolates of *Alternaria* from foods and feeds were lethal when corn-rice cultures were fed to weanling rats. Doupnik and Sobers (1968) grew 96 isolates of *A. longipes* on cracked corn and fed them to 1-day-old chicks. During the 2-week trial, 53 isolates had no apparent effect, 12 depressed weight gains, and 31 were lethal. Toxic effects were noted when *Alternaria*-infested grain was fed to chicks (Forgacs

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and Carll, 1962), when geese were fed culture medium from an *A. tenuis* (*A. alternata*) isolate (Palyusik et al., 1968), and when homogenized *Alternaria* cultures were injected intraperitoneally into mice (Hamilton et al., 1969). Specific metabolites were not mentioned in any of the above reports.

More recently, Meronuck et al. (1972) showed that 20 of 23 *A. alternata* isolates that were lethal to rats produced tenuazonic acid (TeA), and that 11 nontoxic isolates did not. Approximate LD₅₀ values (mg/kg) of purified *Alternaria* metabolites administered by intraperitoneal injection have been determined for mice as follows: AME and AOH > 400, altertoxins I and II about 150, and altenuene (ALT) 75–100 (Pero et al., 1973; Harvan and Pero, 1976). Similar tests by Gitterman (1965) showed an LD₅₀ of 81 mg/kg for TeA. Smith et al. (1968) reported a lethal dose of TeA for female rats was between 137 and 205 mg/kg; Meronuck et al. (1972) found female rats succumbed when given 100 to 200 mg/kg of TeA.

We report the results of tests in which diets containing cultures of four isolates of *Alternaria alternata* were fed to rats and chicks. Amounts of four *Alternaria* metabolites in the diets were known.

MATERIALS AND METHODS

***Alternaria* Cultures.** Four *A. alternata* isolates, three from sorghum grain and one from hard red spring wheat, were selected for feeding trials on the basis of metabolites each produced. A 1:1 mixture of yellow corn and milled rice was dispensed in 350-g lots into 1-L flasks, adjusted to 35% moisture content, sterilized, inoculated, and incubated 3 weeks at 24 °C. Flasks were shaken daily to reduce clumping. The ten flasks of each isolate then were air-dried, ground, and blended together for analytical tests and incorporation into rations.

Weathered Sorghum Grain. Twelve samples of dark-colored grain representing six hybrids and three locations in eastern Kansas were harvested in 1973 from fields managed by the Kansas Agricultural Experiment Station. The samples were selected from lots collected for a study of occurrence of alternariols in grain sorghum (Seitz et al., 1975). Alternariol (AME + AOH) contents averaged 4.8 µg/g and ranged from 2.5 to 7.9 µg/g.

Analytical Methods. AME, AOH, and ALT in culture media were analyzed by high-pressure liquid chromatography as described previously (Seitz and Mohr, 1976). The extracts were also used for detection of altertoxin I (ATX-I) by thin-layer chromatography (TLC). Sorghum grain extracts (Seitz et al., 1975) were analyzed by TLC for ATX-I and ALT. We used Brinkmann SILG-HR-25 (Cat. No. 6614600-6) precoated plates activated 1 h at 120 °C and developed in chloroform–acetone (88:12, v/v). *R_f* values were 0.21 and 0.12 for ATX-I and ALT, respectively. Protein was determined by the Kjeldahl nitrogen method for mixed feeds (AOAC, 1975).

Tenuazonic acid was analyzed in 50 g of sorghum grain or 25 g of ground, dried culture medium. Samples were homogenized with 150 mL of 0.01 N HCl in a blender for 2 min, and the mixture was centrifuged. The supernatant was decanted into a separatory funnel containing 60 mL of 10% (NH₄)₂SO₄ and extracted with two 35-mL portions of ethyl acetate. The combined ethyl acetate extracts were further extracted with two 50-mL portions of 3% NaHCO₃. The NaHCO₃ extracts were combined, adjusted to about pH 2 with 1 N HCl, and reextracted with two 30-mL portions of ethyl acetate. The extracts were dried over anhydrous CaCl₂ and evaporated under N₂ to near dryness on a steam bath. The concentrated solution was quantitatively transferred to a 1-dram vial and evaporated to

complete dryness. The residue was redissolved in 0.5 mL of pyridine that had been dried over anhydrous CaCl₂.

Extracts were analyzed by gas chromatography (GC) for detection and quantitation and by TLC to confirm presence of TeA. For TLC, pyridine solutions were spotted directly on an activated Brinkmann SILG-HR-25 plate. After development in toluene–ethyl acetate–formic acid (5:4:1, v/v/v), TeA was a tan spot at *R_f* 0.65 that turned brownish-orange upon treatment with ethanolic ferric chloride solution (0.25 g/mL).

For GC analyses, the trimethylsilyl derivative of TeA was formed by adding 0.4 mL of *N,O*-bis(trimethylsilyl)acetamide and 0.1 mL of trichlorosilane to the pyridine solution and warming to 50 °C for about 10 min. A Bendix Model 2600 dual-column chromatograph equipped with flame ionization detectors was used. Glass columns, 1.83 m × 6.35 mm o.d. (4 mm i.d.), were packed with Gas-Chrom Q (100–120 mesh) coated with 3% OV-17 in one column and 3% OV-101 in the other. Each column was conditioned 24 h at 250 °C. The detector and injector temperatures were 300 °C and N₂ carrier flow rate was about 25 mL/min. Column temperature was programmed from 125 to 250 °C at 10 °C/min. Tenuazonic acid (trimethylsilyl derivative) eluted at 10.3 and 8.2 min with the OV-101 and OV-17 columns, respectively. The OV-101 column minimized interferences in analysis of sorghum grain.

Standards (usually 1.9 µg/µL, free acid form) were prepared from a purified sample of the *N,N'*-dibenzyl-ethylenediamine salt of TeA kindly supplied by D. J. Harvan and R. W. Pero, National Institutes of Environmental Health Sciences, Research Triangle Park, N.C.

Feeding Trial. Ground corn–rice cultures and uninoculated corn–rice medium were mixed with ground rodent chow or chick-starter ration to constitute either 10 or 50% of the total ration. Chick-starter rations supplemented with 10% corn–rice contained 21% protein; 50% supplemented rations had 17.5% protein. Rodent-chow diets had 24 and 18% protein in the 10 and 50% supplemented feeds, respectively. Each diet was fed ad libitum to a group of four young female white rats and to a group of five 2-day-old broiler chicks.

Animals were observed daily for signs of toxicity and were weighed weekly. Dead or moribund animals were examined for gross lesions and tissues were prepared for histological examination. On day 21 all survivors were sacrificed and completely examined for gross and microscopic abnormalities.

RESULTS

Quantitative analyses of AME, AOH, ALT, and TeA in test diets fed to rats and chicks are shown in Table I; the presence of ATX-I is indicated also.

Effect of *Alternaria* Metabolites on Chicks. Chicks were not adversely affected by substituting a 1:1 corn–rice mixture infected with any of the four *Alternaria* isolates for 10% of a starter ration (Table I). The birds ate well, gained weight, and were alert. No significant post-mortem lesions were found on gross examination of organs or by microscopic examination of tissue sections. When half the diet for chicks was 1:1 corn–rice–*Alternaria* mixtures, average weight gains of chicks on rations B and C were equal to or greater than the gain of birds fed the uninoculated control diet. Lower weights reflect the lower protein content of the diets containing 50% corn–rice.

Three birds fed isolate HRS-5 in ration D-50 had leg weakness and locomotor difficulty and were destroyed on day 3 of the feeding trial. The two survivors were unresponsive and the wings drooped; however, by day 7 both

Table I. *Alternaria* Metabolites in Test Diets and Average Weights of Rats and Chicks during Feeding Trial

diet	<i>Alternaria</i> isolate	corn-rice culture, ^a %	<i>Alternaria</i> metabolites ^b					av wt (g/chick)					av wt (g/rat)				
			AME, µg/g	AOH, µg/g	ALT, µg/g	TeA, µg/g	ATX-I	initial	7 days	14 days	21 days	initial	7 days	14 days	21 days	initial	7 days
			µg/g	µg/g	µg/g	µg/g											
A-10	None	10	0	0	0	0	0	43	76	147	202	168	196	206	218	168	196
B-10	RL-671-2 ^c	10	4.9	4.0	2.1	0	0	43	82	156	214	168	195	204	219	168	195
C-10	RL-8442-3 ^d	10	1.8	7.8	0.08	0	0	43	78	150	190	167	188	192	205	167	188
D-10	HRS-5	10	2.8	0.7	0.04	29	++	41	74	139	198	168	179	190	199	168	179
E-10	FN-8442-5	10	1.9	1.5	0.3	74	+	39	73	145	200	167	193	198	209	167	193
A-50	None	50	0	0	0	0	0	41	78	127	180	168	196	205	221	168	196
B-50	RL-671-2	50	24.0	20.0	10.0	0	0	39	69	130	184	168	191	200	210	168	191
C-50	RL-8442-3	50	9.0	39.0	0.4	0	0	45	79	146	187	168	188	200	210	168	188
D-50	HRS-5	50	14.0	3.5	0.2	145	++	41	40 (2) ^e	70 (2)	92 (2)	170	127	(0)	(0)	170	127
E-50	FN-8442-5	50	9.5	7.5	1.5	370	+	43	48 (2)	70 (2)	87 (2)	166	132	140 (2)	132 (1)	166	132

^a Remainder of diet was standard laboratory animal ration. ^b Each figure is the average of triplicate analyses. ^c ATCC 34958. ^d ATCC 34956. ^e Number in parentheses is number of survivors when fewer than original five chicks or four rats.

were eating and, though small, appeared to have recovered. Ration E-50 containing isolate FN-8442-5 was lethal for one bird on day 3 and two on day 4. At that time one survivor had a stilted walk and both were listless. Birds remained listless throughout the test although they ate and gained weight; the chick recovered from the locomotor difficulty.

At post-mortem examination, all chicks that died during the first week of the test had bleeding erosions in the lining of the isthmus and erosions or pre-erosions in the gizzard with hemorrhages beneath the mucosa. On histological examinations necrosis and hemorrhages were seen in the gizzard and isthmus of birds fed rations D-50 and E-50. Proventricular lesions also were present in some birds fed D-50. The birds surviving the 3-week trial also had lesions of the gizzard and isthmus. Lesions of these organs were not seen in chicks fed any of the other rations.

Effect of *Alternaria* Metabolites on Rats. Rats fed diets B-10, B-50, C-10, and C-50 accepted the rations as well as those fed the uninoculated A-10 and A-50 diets and gained weight (Table I). Necropsy findings, which included examination of the reproductive tracts for estrogenic effects of *Alternaria* metabolites, were negative. The D-50 and E-50 rations were consumed sparingly; D-10 was wasted during the early days of the feeding experiment. E-10 was the only diet containing TeA that was not noticeably refused. The animals fed D-10 were hyperactive after 3 weeks on the ration and weighed less than others fed 10% supplements to rat chow. No post-mortem lesions were observed.

After 1 week rats offered diets D-50 and E-50 had lost weight. No rats survived on D-50; deaths occurred on days 9, 10, and 11. Two animals fed E-50 died on day 11 and another on day 20. Rats on that diet were cannibalized by any survivors, so necropsy and histological findings are incomplete. Hemorrhages were observed in the stomach and intestines.

***Alternaria* Metabolites in Field Samples.** Tenuazonic acid was not detected in any of the 12 samples of heavily weathered grain sorghum although the alternariols were present in quantities nearly as high as those in the 10%-*Alternaria*-isolate supplemented diets. Only three of the 12 samples had ALT at a level higher than 0.10 µg/g estimated from TLC: 0.12, 0.40, and 1.5 µg/g. The same three samples had low intensity, yellow fluorescent spots, indicating traces of ATX-I. The other samples showed no more than very faint ALT or ATX-I spots on the TLC plate. Identification of ALT and ATX-I was confirmed by cochromatography of samples and standards and by the absence of ALT and ATX-I spots in several samples of unweathered grain that did not contain alternariols.

Insufficient quantity of standard precluded testing the recovery of TeA from culture media or sorghum grain by our procedure. A weathered sorghum grain sample was extracted successively with 0.01 N HCl and methanol-0.01 N HCl (90:10, v/v); TeA was not found in either extract. The GC method should have detected TeA in the grain at levels above about 1 µg/g.

DISCUSSION

Culture media of two *Alternaria* isolates that synthesized TeA and ATX-I induced toxic symptoms and were lethal to chicks and rats. The other two isolates that produced AME, AOH, and ALT in different ratios had no deleterious effect, although the amounts of metabolites in the 50% corn-rice rations were about six times greater than those found in heavily weathered sorghum samples (Seitz et al., 1975). These findings and those of Meronuck et al. (1972), in which 20 of 23 toxigenic *Alternaria* isolates

were TeA producers, suggest that TeA is responsible for most of the lethal effect observed in laboratory feeding tests of *Alternaria* isolates grown on grain substrates. However, ATX-I also may have contributed to the toxicity we observed. Isolate HRS-5 was a stronger producer of ATX-I than FN-8442-5 which may explain why D-50 and E-50 diets were equally lethal to chicks, and D-50 was more toxic than E-50 to rats, even though E-50 had the higher TeA concentration. Production of ATX-I by *A. alternata* isolates is not always associated with production of TeA.

Alternariols measured in weathered sorghum grain may have been produced by several separate invasions of the maturing seeds and of mature seeds as they were repeatedly wetted and then dried during rainy periods. Most infections probably were actively growing for only a short time and perhaps not long enough for a late-produced metabolite, such as ALT (Burrough et al., 1976), to accumulate appreciably. If TeA and ATX-I are also late-produced metabolites, this would explain the absence of TeA and the trace amounts of ATX-I in the weathered sorghum samples. Meronuck et al. (1972) did not find TeA in a sorghum sample heavily invaded by *Alternaria*. There have been two reports of TeA in crops: in rice plants (Umetsu et al., 1973) and in tobacco plants (Mikami et al., 1971). The steady, high moisture contents of susceptible green plants permits continuous growth of the fungus for many days.

Although no toxic effects were observed from feeding *Alternaria* isolates that do not produce TeA or ATX-I, results of a short-term feeding trial do not answer questions about the effects of long-term ingestion of grain heavily invaded by *A. alternata*.

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Aflatoxin Inactivation in Corn by Ammonia Gas: Laboratory Trials

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An ammonia (NH₃)-air mixture was recycled at 25 °C through a glass column containing aflatoxin-contaminated, shelled corn until uniform distribution of NH₃ in the corn bed was achieved. After ammoniation and sealed storage at 17.6% moisture and 25 °C for 14 days total reaction time, aflatoxin B₁ level was reduced from 1000 µg/kg (ppb) to 10 µg/kg. Effects of varying the NH₃ addition level, corn moisture, reaction time, initial aflatoxin level in corn, and recycle gas flow rate were investigated. A pilot-scale experiment wherein 4.86 metric tons of 11% moisture corn was treated with 1.1% of NH₃ reduced the corn's B₁ level from 90 µg/kg to a nondetectable level during 7 months of storage indoors. Swine feeding tests on this ammoniated corn gave good results as reported elsewhere. In laboratory experiments, aqua and gaseous NH₃ appeared equally effective for inactivating the aflatoxin in corn.

Various acids, alkalies, oxidizing agents, aldehydes, and nitrogen compounds have been tested for the ability to chemically inactivate the aflatoxin present in contaminated feeds and foods (Dollear, 1969; Mann et al., 1970; Detroy et al., 1971; Goldblatt, 1971; Beckwith et al., 1976). For large-scale detoxification, the chemical with greatest practical potential appears to be ammonia (NH₃). NH₃ inactivation of the aflatoxin in oilseed meals, particularly

cottonseed meal, has been reported by Masri et al. (1969), Dollear (1969), Mann et al. (1970, 1971), Gardner et al. (1971), and McKinney et al. (1973). For pilot- and plant-scale tests on cottonseed meal, NH₃ gas under pressure and elevated temperature (typically 30 min at 110–120 °C and 310–345 kPa, i.e., 45–50 psig in the plant test) was used.

This paper reports on laboratory experiments completed at room temperature to study the effect of several process variables upon the degree of aflatoxin inactivation obtained by treating a stationary bed of corn with a recycling mixture of NH₃ and air as proposed by Lancaster et al. (1975). Process variables investigated included: ammonia

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